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MOLECULAR BIOLOGY AND AGRICULTURE

Potential for Future Research

Report of
Western Experiment Station
Collaborators Conference
March 13—16, 1968,
Albany, California

Agricultural Research Service
United States Department of Agriculture

EACH YEAR THE REGIONAL UTILIZATION RESEARCH DIVISIONS OF THE U.S. Department of Agriculture hold conferences attended by representatives of State Agricultural Experiment Stations in their regions.

These conferences of agricultural scientists fulfill needs for reports and discussion in areas of special importance. In the western region the subject in 1967 was Proteins for Aid and Trade; in 1966 it was The Destruction of Salmonellae.

In 1968 (March 13-15) the subject was Molecular Biology in Agriculture: Potential for Future Research. Molecular biology is in certain respects a new scientific discipline, created within the past two decades by deeply penetrating biochemical discoveries in the area of genetic processes. Within the U.S. Department of Agriculture this conference was the first to be exclusively devoted to molecular biology.

The program was planned by Alfred C. Olson, Chemist in the Plant Enzyme Pioneering Laboratory of the Western Utilization Research and Development Division, and Paul K. Stumpf, Chairman of Biochemistry and Biophysics in the University of California at Davis.

The plan used in reporting the conference is explained in a note on the following page. Occasional use of commercial names for materials does not imply recommendation by the U.S. Department of Agriculture.

The data, illustrations, references, and the style used in references are those supplied by the speakers.

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Western Utilization Research and Development Division
Agricultural Research Service
UNITED STATES DEPARTMENT OF AGRICULTURE
Albany, California 94710

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Some explanation of the arrangement of materials in this report is necessary. Those who planned the conference realized that the contributors are scientists engaged in research in segments of the general area of molecular biology. Their contributions, which are presented in summarized form, reveal problems and discuss research methods and advancements.

The need for discussion directed toward review and perspective also seemed apparent. This need has been appropriately met by remarks authored by James Bonner, Professor of Biology in the California Institute of Technology, and presented by him informally. Salient extracts from his remarks are included here. For convenience, they are placed first.

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MOLECULAR BIOLOGY AND AGRICULTURE

POTENTIAL FOR FUTURE RESEARCH

BACKGROUND AND OUTLOOK

James Bonner
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(Two portions of Dr. Bonner's remarks are included below. The first one is a statement of present status. The second is a longer excerpt from remarks that were recorded on tape and transcribed.)

Present Status

During the first half of this century, two of the major branches of agricultural research, breeding and utilization, have been nourished and guided by the sciences of genetics and biochemistry, respectively. Just as the new science of molecular biology has wedded these two disciplines into a penetrating understanding of life, so may we expect that it will erase all barriers between them.

At the core of molecular biology is DNA (deoxyribonucleic acid), the genetic substance. Its role as the master template for the functioning and reproduction of cells of living organisms is now well established. Molecular biology tells us that the synthesis of enzymes required for this functioning is under the control of, and is dictated by, the sequence of nucleotides in the DNA.

Less well known is the method by which cells differentiate and become specialized. Although all cells of a given organism contain the same genetic code (the same DNA) the expression of much of the code in the cell of a given organ at a given time is guided by represser substances. In bacteria these repressers are specific proteins which combine with those specific regions of DNA that are to be repressed. In higher organisms such as plants, the repression is effected by nonspecific proteins, histones, to which are attached short RNA (ribonucleic acid) chains. Recent research indicates that specificity of repression lies in the nucleotide sequence of this histone-associated RNA. Histones can be selectively removed from the DNA, thus "turning on" specific genes.

Thus the pathway of differentiation and development of a given cell is dependent upon its local environment. Recent experiments show that development and differentiation can be artificially influenced (i.e., the chromosomes can be reprogramed). These investigations suggest new ways to endow plants with desired genetic characteristics, the possibility of creating a completely new plant species, or a way to make identical copies of one plant. Such novel and still unachieved approaches do not mean that advancement in plant and animal breeding by traditional means will soon be exhausted. Dwarf wheat and high-lysine corn are results of traditional methods and undoubtedly more similarly excellent achievements will be forthcoming.

Background and Potential

A cell lives and multiplies because within that cell is a book of instructions on how to make everything that's in that cell. This "book" is DNA or deoxyribonucleic acid. It is an extremely large molecule made of four kinds of building blocks or nucleotides called adenine, thymine, guanine, and cytosine, commonly designated by their initials, A, T, G, and C. DNA is able to replicate and it's the only molecule that has that power.

One of the greatest triumphs of modern biology was the fairly recent discovery of the mode of replication of DNA. Each molecule consists of two chains wrapped around one another. The first is mated with a companion chain, also made of A, T, G, and C. The two chains are mated in accord with a special law called complementarity. This law says that where there's an A in chain No. 1, there must be a T in chain No. 2. Where there's a T in chain No. 1, there must be an A in chain No. 2. Where there's a G in chain No. 1, there must be a C in chain No. 2. And where there's a C in chain No. 1, there has to be a G in chain No. 2. Each cell contains a pool of the nucleotides. The two chains draw on the pool. When the monomers have piled up on the templates the DNA polymerase connects them. The chains separate and we have two molecules, each identical with the original. Now the cell is free to divide and give to each daughter a copy of the genetic information.

All other parts of the cell are concerned with providing a supply of A, T, G, and C. The DNA causes A, T, G, and C to be made by enzymes which use the available food. DNA also makes another kind of long-chain molecule, called ribonucleic acid or RNA. RNA consists of four kinds of building blocks that are analogous to A, T, G, and C. They are called A', U', G', and C'. The U stands for uracil, which resembles thymine. The cell contains a pool of these building blocks. In their presence,

the double-stranded DNA becomes temporarily single stranded and the monomers align themselves along the single-stranded chain. U' pairs with A, A' pairs with T, C' pairs with G, G' pairs with C.

When that assemblage is completed and the monomers are arranged on the DNA template, an enzyme, a second kind of polymerase, connects them. We now have an RNA molecule. Then the hydrogen bonds which held the RNA to the DNA break. Those that held DNA together are reestablished and the RNA molecule is now free. RNA molecules cannot replicate themselves. They have one great power which DNA molecules do not have. RNA molecules can direct the synthesis of enzyme molecules.

How does this work? The DNA molecule is long, corresponding to a million "letters" or more. The RNA molecules are short. The RNA represents the information in one genetic unit of the DNA. It is a small section of the DNA, corresponding to one or two thousand letters. The "letters" are coded instructions on how to make a single kind of enzyme molecule. One kind of RNA transcribed from DNA, for example, will direct the synthesis of peroxidase while another will direct the synthesis of α-amylase.

How is this information used? An enzyme molecule is made of amino acids strung together in a linear chain. The kind of enzyme is determined by the sequence of the 20 kinds of amino acid monomers. The enzyme molecule, on the average, is a hundred or a few hundred amino acids long, and contains the message units necessary to synthesize enzymes, including directions to start the synthesis, to add amino acids in a definite pattern, and finally to terminate the molecule.

The RNA message is translated by a large enzyme molecule, a super enzyme molecule, called a ribosome. There are about 5-50 million ribosomes in a typical cell. The messenger RNA molecule fastens to the ribosome, which then translates each message unit. It moves bodily down the messenger RNA molecule reading what it says, putting things together, and when it comes to the end it sends the finished enzyme out to work. In a typical cell of a higher organism like ourselves, there are about ten thousand kinds of enzyme molecules and there are, on the average, about 50 thousand individuals of each kind. Thus only one kind is a very small fraction of the total.

During the past several years, it has become possible to isolate the chromosomes from higher organisms. When isolated chromosomes are supplied with A', U', G', and C' and the polymerase enzyme, they make messenger RNA molecules just as they do in life. And it is possible to show by experiment that in isolated chromosomes the same genes are turned on for the making of

messenger RNA, as in life, and the same genes are also turned off as in life. Using isolated chromosomes we can try to find what it is that turns on some genes and turns other off.

We know what the material basis is. We know that of the many kinds of proteins in a cell, there is one whose duty is to be wrapped around the DNA. This kind of protein is found only in the chromosomes of higher organisms. Everything that has a nucleus has these proteins, which are known as histones. When DNA has histone protein wrapped around it, the polymerase enzyme cannot transcribe it, cannot make a messenger RNA molecule. Thus in the cells of one part of a differentiated creature, certain parts of the DNA are not covered with histone. Here RNA and enzymes can be made. In another part, these portions of the DNA are covered with histone and cannot be transcribed and the genes are turned off.

Very recently we found out how to remove histones from the DNA. The molecules of histone are attached to the DNA by a little piece of nucleic acid. Proteins can't read the sequence of letters in a DNA very well. They must read the letters by a kind of base pairing, or base complementarity. Each of the protein molecules that interacts with a specific gene of DNA to repress it has a little piece of RNA which, by the strategy of U' pairing with A, A' pairing with T, C' pairing with G, etc., finds its complementary place in the DNA, and sits down and deposits histone molecules on the gene. These RNA molecules are 40 nucleotides long in plants and peas and about 60 nucleotides long in rats, cows, and humans. We can separate the histone molecules from the associated DNA of one organ and make a solution of them. We can also take purified DNA from a different organ of the same organism and mix the two--put them back together. For example, we can take liver histone proteins and muscle DNA, mix them, let them rejoin, and thus make chromosomes that are identical with the liver chromosomes. Now we are learning something about the way in which DNA molecules are specifically repressed. We have a long way to go to find out how nature removes histones and puts them back but we're making progress.

Let's consider next how differentiation might occur. When we take some cells from a higher organism, like a carrot, and grow them in tissue culture, they grow just like bacterial cells. If we take a single cell from such a culture and put it in nutrient medium containing chemical substances that are normally in the embryo sac, or the sac in which plant embryos normally develop, the cell develops into an embryo, and ultimately into a whole plant. Thus single cells multiply and develop into embryos when they are placed in the appropriate media. In this way one can make literally millions of embryos or millions of adult plants

with exactly the original constitution. We now have a way of resetting the developmental program of a mature plant cell that would normally never divide again.

We can't do this with animal cells yet. A related thing, however, can be done with animals. The nucleus of the egg of a Xenopus toad can be either removed or inactivated. The nucleus can be scooped out of the egg or killed with an ultraviolet beam so that it can't work. Next we take a specialized cell from a Xenopus tadpole, such as a cell from the epithelial lining of the gut. This adult cell, containing a nucleus, would normally never divide again although it still makes RNA, ribosomes, and enzymes. The nucleus of this cell can be transplanted into the previously prepared egg. Within a few minutes it starts to behave like the nuclei in the tissue culture experiment I mentioned before. The nucleus "discovers" that the egg contains compounds that make this an egg. The compounds and cytoplasm of the egg interact with the represser molecules and the DNA of the chromosomes of the transplanted nucleus and bring about the situation of genes turned on and genes turned off, appropriate for a fertilized egg. This egg now develops into an adult toad. Thus, we can make any number of creatures that are identical in genetic constitution.

I don't doubt that this kind of information will be useful in making new plants and animals. By breeding procedures it is impossible to make animals of identical genetic constitution. It is now possible to use a kind of vegetative reproduction with animals as we have with some kinds of plants, for example, date palms, for thousands of years. These simple examples show that we can reprogram the chromosomes of an adult organism to fertilized egg time. With increasing knowledge we may be able to reprogram the chromosomes of an adult cell to any desired spot in the developmental history--or into new lungs or hearts or other desired organs.

You might inquire whether molecular biology has been able to transplant a chromosome or a piece of a chromosome from one organism into a second acceptor, as a means of transferring genetic information. This has been possible so far only with the bacteria.

These brief remarks provide us with a background. As our understanding of these basic processes increases, so will gains be made in applied agriculture. The dramatic successes of plant and animal breeding programs of recent years might be surpassed if experiments currently in progress in making crosses between species prove successful. In this way I would envisage for example

the addition of the nitrogen fixation character to, let us say, the cereal grains. In this new technology we have for the first time a way to get around nature's barrier to crosses.

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THE CONFORMATION OF NUCLEIC ACIDS IN SOLUTION

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The important primary, secondary, and tertiary features of DNA have been known for a considerable time (1) and an extensive knowledge of the same features for RNA has developed in the past decade (2). Studies of conformation or of conformational changes of nucleic acids have been made using almost every known physical and biological technique (3). The four factors which contribute to the conformations of nucleic acids are: the structure of the monomeric unit, hydrogen bonding, base stacking, and the chemical environment (i.e., the solvents and ions present).

A recent article by Haschemeyer and Rich (4) considers conformation of nucleosides theoretically by calculation of the contact interactions of the atoms through use of crystallographic data for their bond lengths. Under consideration is whether the

heterocyclic base is syn or anti with respect to a plane passing perpendicularly through the C_1, - O_1, bond; whether the C_2, or C_3, atom is out of plane and in what sense, exo or endo. These workers concluded that the predominant form for the major nucleotides is anti, in agreement with other work (5). No conclusion was reached concerning the furanose ring conformation, even though only one conformation should be allowed in a biologically active macromolecule.

Model systems of di- and tri-nucleotides have been used to demonstrate hydrogen bonding (6) and base stacking. Nuclear magnetic resonance has been used recently in these fields (8), and provides one of the most graphic examples of stacking in the "near neighbor effects" shown by the thymidine methyl group (8). Studies of the environmental factors influencing oligo nucleotide ordering are now receiving much attention (9,10). Fresco et al. have been able to correlate conformation changes in s-RNA with amino acid acceptor activity, and to show the great importance of metal ions to the active conformation (2b). The melting curves obtained by these workers for s-RNA show an initial melt between 20° and 40° when monitored by sedimentation value or viscosity, which does not appear when monitored by O.D. changes. It is interesting that the n.m.r. data of McDonald, Phillips, and Penswick (8c) would be in accord with the former results. High-molecular-weight RNA (and single-stranded DNA) has been postulated to have organized double stranded helical regions and disorganized loops or folds (2a). That organized structure is present even in synthetic polynucleic acids is clearly seen in the work of Michelson and associates (10) and other workers using synthetic and chemically modified polynucleotides (11). Michelson can demonstrate differential reactivity of polynucleotides, depending on whether they are single or double stranded, and by changing the reaction conditions. The effect of chemicals on the conformation of nucleic acids in solution has special importance in mutagenic studies involving TMV, as has been recognized by Singer and Fraenkel-Conrat (12a). This is easily seen by the variety of conditions TMV-RNA is subjected to in mutagenic studies (12a,b,c). A clear case of conformational effects involving TMV-RNA can be shown by chain-breakage studies involving the reaction of non-aqueous diazomethane (13). TMV-RNA stays largely intact until a very large excess of diazomethane is employed; then it is extensively degraded. In view of the facile degradation of dinucleotide phosphates by diazomethane (14) it is surprising that TMV-RNA remains intact as long as it does. This appears to be a case where conformation is stabilizing the nucleic acid.

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TEMPLATES IN A TEST TUBE: ENZYMATIC REPLICATION OF DNA

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Topics that illustrate accomplishments in the field of enzymatic replication of DNA and its possible role in agriculture are: the discovery and isolation of DNA polymerase; the role of this enzyme in the putative "creation of life in a test tube"; and studies carried out in the laboratory of Dr. James Bonner at California Institute of Technology on the mode, enzymes, template (nucleohistone), and herbicidal inhibition of DNA synthesis in the cells of higher plants.

Although the elucidation of the structure of DNA by Watson and Crick in 1953 (1) led to a logically deducible model for gene

replication, there was no enzymological or experimentally proved basis for this model until the discovery and purification of Escherichia coli DNA polymerase in 1958 (2). The chemical mechanism was shown to involve a nucleophilic attack of the 3'-OH of the last deoxyribose of a growing polydeoxynucleotide on incoming deoxynucleoside triphosphate to form a phosphodiester bond with the liberation of pyrophosphate. The requirements of the reaction and most of the properties of the newly synthesized DNA fulfilled most of the requirements for a true replication from DNA template. However, in contrast to the Watson-Crick model and in vivo observations, the polymerase did not catalyze synthesis of complementary strands of DNA from the same end of a linear double-stranded DNA template (Y-type replication). This resulted in a highly branched DNA product which was undenaturable and had unusual electrophoretic properties (4). Some of the DNA synthesized at high template concentrations appeared to be single-stranded (5). Furthermore, the product DNA did not possess biological activity. These observations suggested that the polymerase was a "repair" enzyme rather than a true replicase. This problem was circumvented by the use as template of single-stranded circular DNA of the bacterial virus ϕX 174. With the aid of auxiliary enzymes (including the newly discovered DNA "joining enzyme" (6)) it was possible to synthesize and isolate in a test tube, the infectious chromosome of the bacteriophage with its complete set of genes from nonliving chemicals (7).

The Watson-Crick model of replication predicts that the DNA of the first generation of daughter cells in a growing culture of cells will consist of a hybrid containing parent and newly synthesized DNA (semiconservative mode of replication). By growing tobacco cells in an N-15-containing medium and then transferring to an N-14-containing medium and determining the sedimentation characteristics of the isolated DNA therefrom, it was shown that the mode of replication of DNA in higher plants (like that of bacteria and animals), is semiconservative (8).

After several false leads (9), DNA polymerase activity was demonstrated for the first time in plant tissue, the roots of the mung bean seedling (10). Although the specific activity of crude extracts is about 0.1 percent that of bacteria, the activity of DNA polymerase per unit weight of DNA is the same for both plants and bacteria. This illustrates the general principle that a cell will make just enough enzyme in response to its requirement for substrate or product.

The natural template for DNA synthesis in plants is the DNA contained in the chromosomes of the nucleus of the plant cell. The chromosomes of the pea embryo can be prepared and isolated as a preparation of chromatin which consists largely of DNA partially complexed to basic proteins known as histones (11). Chromatin,

because of its free DNA, can serve as template for messenger RNA synthesis, which in turn directs enzyme (protein) synthesis. However, the fraction of chromatin DNA covered with histone (nucleohistone) cannot serve as template for RNA synthesis. Thus histone plays a role in the regulation of the gene expression by repressing those genes not needed at a given time in the life cycle of the plant. Nucleohistones can serve however, as template for DNA synthesis by DNA polymerase (12). Furthermore reconstituted nucleohistones, prepared by combining free DNA with various histone fractions, can also support DNA synthesis (13).

From studies on polymerase kinetics (14), susceptibility of product DNA to certain nucleases (15) and from ultracentrifugal and electrophoretic properties of the newly synthesized DNA (16), it is concluded that plant nucleohistone supports limited synthesis of double-stranded, unbranched DNA, that most of the DNA product is complexed with the template (but not in the same manner as the DNA of the template is complexed with histone), and that there is very little transfer of histone from template to product. These data are in agreement with the view that histones are conserved during cell division.

The herbicide 2,4-dichlorophenoxyacetic acid inhibits the activity of DNA polymerase at 10^{-5} M (17). This inhibition may contribute to the toxic effect of this herbicide, by preventing or slowing down cell division.

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RNA TRANSCRIPTION

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The transcription of RNA from the DNA template is catalyzed by RNA polymerase. This enzyme was first demonstrated in rat liver nuclei by S. B. Weiss (1) and subsequently extensively purified from several bacterial species including E. coli (2), M. lysodeikticus (3), and A. vinelandii (4). The in vitro characteristics of RNA polymerase are similar; for RNA synthesis RNA polymerase requires a divalent cation (Mg^{++}, Mn^{++}), ribonucleoside triphosphates (ATP, UTP, GTP, CTP) and a template (5). The template in vitro is not limited to native DNA, since single-stranded DNA and polyribonucleotides will also direct polynucleotide synthesis (6). In addition to template-directed reactions RNA polymerase will also catalyze two unprimed reactions leading to poly A:poly U homopolymers (7) and rIC copolymer (8). The unprimed reactions

demonstrate the complementary nature of the RNA polymerase reaction, since the products obey the Watson-Crick rule of hydrogen bonding between A and U (or T), and I (or G) and C. This complementarity, by virtue of the specificity elicited by hydrogen bonding, is seen for all RNA (and DNA) polymerase reactions. RNA synthesized in the DNA-directed reaction is complementary in composition and sequence to that of the template; this has been shown by determination of base composition, nearest neighbor frequencies (5) and hybridization (9).

RNA synthesis *in vivo* is known to be asymmetric; that is, within each operon only one strand of the DNA duplex is transcribed into RNA. This is due to the initial event in RNA synthesis; RNA polymerase binds to DNA at specific sites, resulting in a DNA-enzyme complex. From the work of Szybalski and coworkers (10) it would appear that these binding sites consist of pyrimidine-rich tracts on the DNA molecule. In an extreme case, that of T7DNA, these tracts are dC-rich and are all in one strand of the DNA duplex. Therefore it is this strand which is transcribed into RNA. Szybalski has suggested that the dC- and dT-rich regions represent a part of each operator region. Since the represser binds to the operator region, the presence of represser molecules would prevent formation of the DNA-enzyme complex and thereby block transcription of that operon. The DNA-enzyme complex can be demonstrated by sucrose density gradient centrifugation (11) and by retention of the complex on cellulose nitrate filters (12).

Initiation of RNA synthesis occurs by incorporation of an intact ribonucleoside triphosphate to form the 5' end of the RNA chain. This is the first nucleotide incorporated and chain elongation occurs in a 5' → 3' direction (13). Initiation occurs predominantly by incorporation of ATP and GTP and only to a small extent (less than 5 percent) by CTP or UTP (13,14). The GTP/ATP ratio varies with the DNA template used and may reflect the ratio of dC- and dT-rich promoter regions to the RNA polymerase binding sites on the DNA. RNA polymerase binds to the promoter site on the DNA and causes a localized melting of the helix. As polynucleotide synthesis progresses the RNA is found in the form of a transient RNA:DNA hybrid. As polymerase moves along the template the DNA helix reforms and displaces the RNA from this hybrid intermediate.

RNA synthesis *in vitro* does not show linear kinetics and after 60 minutes at 37° the reaction plateaus and no further RNA is formed. This has been shown to be due to product inhibition whereby the RNA synthesized presumably "clogs" the template site of polymerase. It is possible to obtain nearly linear kinetics

by running the reaction in the presence of ribonuclease to hydrolyze the RNA as it is formed and using PP_i released as a measure of polymerase activity (15).

It is an accepted fact that *in vivo* synthesis of protein and RNA are coupled. Stent (16) has suggested a model whereby translation and transcription are coupled by the movement of the ribosomes across the messenger RNA; this "strips" the RNA off the polymerase and prevents product inhibition from occurring. Such a system can be set up *in vitro* and it has been shown that the addition of ribosomes competent in binding RNA will stimulate RNA polymerase (17).

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REGULATORY MECHANISMS INVOLVING ENZYME FUNCTION, SYNTHESIS AND DEGRADATION

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Since the essence of progress in agriculture is learning how to maximize desirable and minimize undesirable biological characteristics, it is important to understand the natural mechanisms by which the characteristics are regulated. All biological characteristics are the result of the combination of enzyme-catalyzed reactions, and so it is appropriate to focus attention on the mechanisms by which catalysis is regulated. There are thirteen points between the gene and the enzyme-catalyzed reaction at which specific control of the reaction rate can be exercised (fig. 1).

The work of Bonner, Huang, and Gilden (1) indicated that the synthesis of pea-seed globulin is controlled by the accessibility of the DNA region in which the amino-acid sequence of pea-seed globulin is encoded. Chromosomal material isolated from the globulin-synthesizing tissue, the cotyledon, could serve as template for the synthesis of m-RNA, which in turn could be translated in pea-seed globulin, while a nonsynthesizing tissue, the pea bud, yielded chromosomal material with far less, if any, of this activity. Deproteinized DNA from either source served equally well as template for synthesis of pea-seed globulin messenger.

Experiments of Leaver and Key (2) have shown that the increase in protein synthesis seen in aged carrot root discs is accompanied by the formation of polyribosomes, which have greater amino acid incorporating activity in vitro. The formation of these polyribosomes was dependent upon the synthesis of an RNA which could be inhibited by actinomycin D, presumably an m-RNA. These results

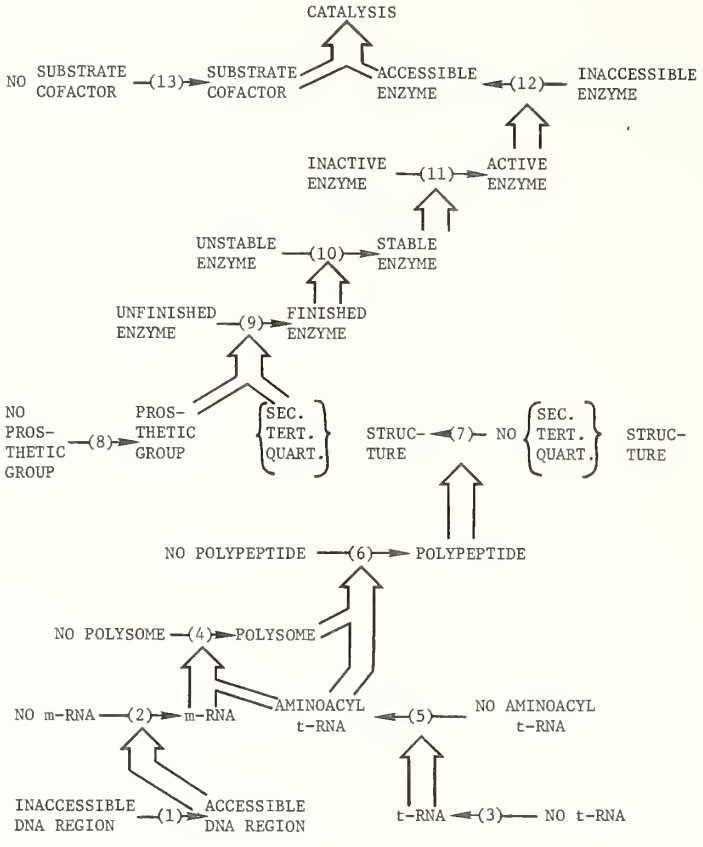

Figure 1. Points of control of reaction rate.

indicate that step 2 is an important regulatory point. The work of Marcus and Feeley (3), however, has indicated that synthesis of m-RNA is not necessary for the initial increase in protein synthesis during wheat embryo imbibition. They were able to activate the protein-synthesizing capacity of extracts of unimbibed embryos, the result being polyribosome formation. In this system, step 3 is the control point.

The importance of t-RNA as a potential point of specific regulation is discussed by Doi elsewhere in this report, based on his work on t-RNA's of sporulating B. subtilis.

When a general stimulation of protein synthesis occurs, as in the aged tissue disc, or the imbibed embryo, the question arises: Is the stimulation specific for any proteins, or are all proteins synthesized more rapidly? Patterson and Trawavas (4) have recently reported data that indicate that indole acetic acid induces the synthesis of specific proteins in pea-stem segments.

The only case in higher plants where specific induced enzyme activities have been shown to be synthesized de novo are the α-amylase and protease induced by gibberellic acid in barley aleurone (5,6,7,8).

Enzyme degradation or stabilization (regulatory step 10) has received attention by a number of people who have studied the control of mammalian liver enzymes. Tryptophan pyrrolase, for instance, increases in level in rat liver in response to tryptophan injections because tryptophan stabilizes the enzyme without altering the rate of synthesis (9). The level of tyrosine transaminase in rat liver is unaffected by cycloheximide because this inhibitor blocks both its synthesis and its degradation (10). Prednisolone induces an increase in alanine transaminase in rat liver by accelerating both synthesis and degradation, but synthesis is accelerated relatively more (11).

An example of the use of regulatory step 11 is in the work of Pressey (12), who has isolated and purified a protein from potato tubers which specifically inhibits invertase. This is particularly noteworthy, since a number of workers are studying the invertase activity which develops during aging of storage tissue discs (13,14).

The RNAse induced by gibberellic acid in barley aleurone cells (15) is accumulated in the cells and is later secreted. Thus we see that step 12 is also used to regulate catalytic activity.

One case in which the study of enzyme regulation has resulted in some suggestive leads for future developments in agriculture is the effect of the herbicide simazine on nitrate reductase (16). Subtoxic levels of simazine cause an increase in nitrate reductase. Plants growing on nitrate plus simazine accumulate more protein, up to 80 percent more, than plants on the same level of nitrate (17). On the other hand, simazine has no effect on the protein content of plants growing on ammonia. Recent work has shown that simazine also increases nitrate and asparagine accumulation (18). It appears from these results that the activities of nitrate permease and nitrate reductase are the limiting factors in protein synthesis on low levels of nitrate, and that simazine increases the rates of these two enzyme-catalyzed processes in a manner which is not yet understood, but which has the desirable effect of increasing the protein content and hopefully the nutritional value of the plant.

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CELLULAR ORGANIZATION

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In intact organisms the biochemical reactions we have been discussing take place in cells, which subdivide all known kinds of living matter into small closed compartments. With the exception of bacterial cells, all other cells are further compartmentalized to provide smaller reaction chambers, where reactants and enzymes meet. The walls of these chambers are thin membranes whose predominantly lipid character renders them relatively impervious to aqueous solutions and hydrophilic molecules. The cell's aqueous phase is thus divided into volumes having a limited and controlled intercommunication. Biological membranes also have a protein moiety, probably enzymatic, and on this constituent depends the second important property of membranes, their selective permeability, which creates within their enclosures a special microenvironment by controlling the ingress and egress of metabolites and ions.

It is apparent then, that viewed as a chemical machine, the functioning of a cell is not determined solely by the enzymes coded in its genome. Enzymes and substrates are not just lumped together. Structural segregation plays a role and structure is largely a matter of membrane topology.

Cells are very small (mostly 20-30 μ in diameter) and can be studied only with the aid of microscopes. Their internal details in fact can only be visualized effectively by means of the electron microscope. The great post-war renaissance of morphology stems from the perfection of this instrument and of methods of processing biological materials for it.

All cells have an external or plasma membrane (fig. 1, PM) which defines their limits, holds their bits and pieces together and is the first outpost of the physiological system creating and maintaining the special internal environment. In addition most large cells have an inner chamber or nucleus in which, except at division, the genetic apparatus is segregated from the rest of the cell (fig. 1B). Bacteria, the smallest and simplest of cells, lack the nuclear compartment (fig. 1A).

In the cytoplasm of nucleated cells are other small membrane-bounded organelles having special functions: mitochondria and in plants chloroplasts and other plastids. Since other speakers will discuss these organelles, they may be passed over here. In any case, fortunately, they behave as almost independent units.

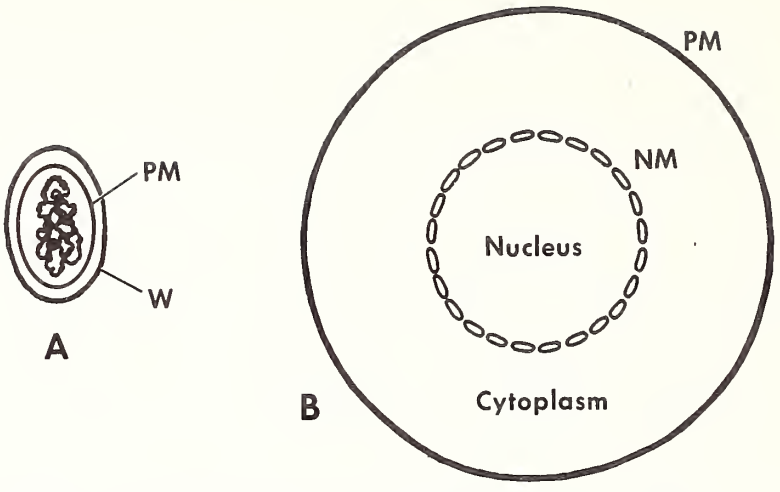

Figure 1. The primary classification of cells: (A) The protokaryote or one-envelope cells. The genetic apparatus lies within the cytoplasm and is not enclosed by a second nuclear membrane (bacteria and blue-green algae). (B) The eukaryote (two envelope cells). The genetic apparatus is segregated from the cytoplasm by a double-layered nuclear membrane (NM). PM is the plasma membrane and W is the cell wall.

Other important constituents, which also can only be mentioned in passing, are: microtubules, an intracellular skeletal component used extensively to stiffen the cytoplasm where needed and to build the mitotic apparatus, the shafts of cilia, sperms, and other elongated protrusions; and centrioles, small, still mysterious objects with the property of organizing microtubules.

Most cellular activities are mediated by biochemical reactions catalyzed by enzymes. A more or less extensively developed system of membranes is devoted to the task of collecting enzymes, channeling them into special compartments, bringing metabolites to these enclosures, and managing the products of their action. This membrane system is the endoplasmic reticulum (ER). So important is this organelle that we are able to describe much of the cell's total activity in terms of its many manifestations. The first steps in protein synthesis up to polypeptide assembly on ribosomes (transcription and translation) are the same in all systems. Membranes play a small role here. But from the moment a polypeptide leaves a ribosome, its fate is in the hands of the ER.

The ER divides the cell space into two parts: inside the closed membranous system and outside (fig. 2). Ribosomes are (with the nucleus and the genetic molecules) outside the reticulum and a fundamental branching of the flow of newly synthesized proteins is established by the dividing membrane itself.

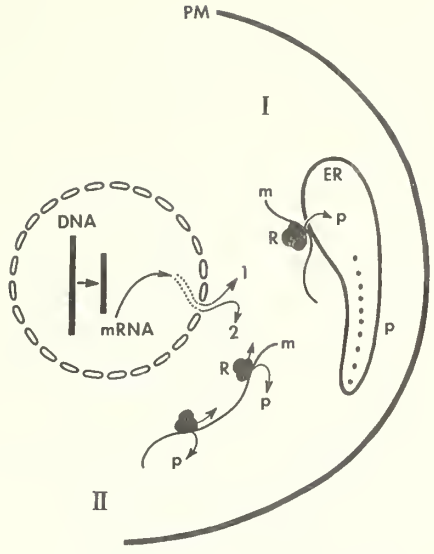

Figure 2. The two pathways of protein synthesis: I and II. Beginning each pathway a nucleotide sequence of the nuclear DNA corresponding to a gene sheds a messenger RNA (mRNA) which passes out of the nucleus, probably via a pore in the nuclear membrane NM, into the cytoplasm. In the cytoplasm the mRNA (according to its code) will either: (I) pass to an element of the rough ER where, associated with a membrane-bound ribosome, it assembles a polypeptide which will be collected within the sac of the ER and be isolated from the cell sap; or (II) pass to a free ribosome where the polypeptide assembled will be shed freely into the cytoplasm and remain the cell sap. PM = plasma membrane, NM = nuclear membrane, R = ribosomes, M = messenger RNA, p = polypeptide, P = protein.

Polypeptides, synthesized on ribosomes attached to the membrane, pass through it and are collected in closed sacs of the ER (fig. 2 I); those formed on "free" ribosomes pass directly into the cell sap outside the membrane system (fig. 2 II). We thus distinguish "free proteins" and "membrane enclosed proteins."

In multicellular organisms cells are specialists, which means they do one main job and they do this by synthesizing one (or a few) enzymes or structural proteins. Such cells are said to be differentiated and their cytology reflects this fact. In some, free ribosomes predominate and the proteins collect in the cell sap to form specialized structures or accumulations on which the differentiated function depends. For example the haemoglobin in red cells converts them into oxygen transports; the fibrous proteins in muscle cells are the basis of contractility; the toughened proteins of epidermal cells form a protective coat. In other cells a variety of functions are based on proteins

originating from bound ribosomes and therefore enclosed in branches or vacuoles of the ER. These activities (summarized in fig. 3) usually involve the participation of an important organelle, the Golgi apparatus, which may be regarded as a specialized region of the ER not associated with ribosomes (fig. 3 at G).

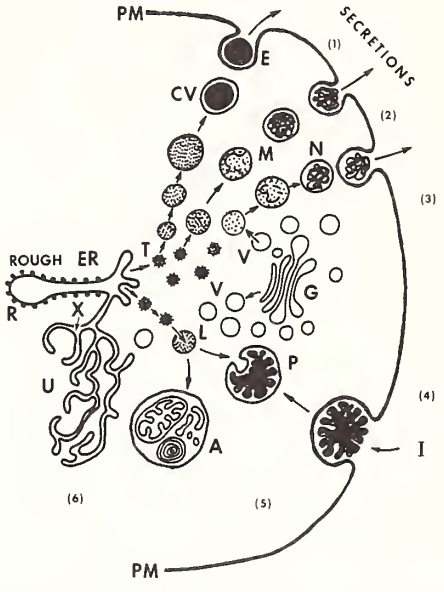

Figure 3. Intracellular movements and Golgi involvement of proteins synthesized on the rough surfaced ER. Note: The diagram is a composite of several pathways not all seen in one cell. Each may be elaborated and become the dominant structural and functional feature of a differentiated cell. In each case the protein is collected from the ribosomes in a sac of the ER from where, according to its composition and role, it may follow any of the several courses: (1) Extracellular secretion of enzymes, etc. The protein is transferred by small vesicles to condensing vacuoles (CV) derived from the Golgi (G) and is passed ultimately through the plasma membrane PM at E. (2 and 3) Enzymes polymerizing polysaccharides move to Golgi vesicles (M and N) where the monomers enter and are polymerized. In (2) polysaccharide only is formed; in (3) polysaccharides are linked to a protein to form mucopolysaccharides (mucins). (4 and 5) Hydrolytic, degradative enzymes collect in Golgi vesicles to form lysosomes L. These may be used either (4) to digest the contents of food vacuoles (P) formed by phago- or pinocytosis or (5) to digest damaged parts of the cell itself which have been walled off in an autophagosome A. (6) Various synthetic enzymes may find their substrates collected in vesicular or tubular extensions of the ER (V) e.g., steroids and lipids synthesis.

Cells, synthesizing proteins for export, e.g. the pancreatic acinar cells, pass their enzymes from the ER sacs in small vesicles to larger vesicles shed from the Golgi (fig. 3 at 1) where they are condensed to granules. Since these are still enclosed in membranes, they can escape from the cell by the fusion of the vacuole membrane with the plasma membrane as at figure 3, E. Very similar events occur with enzymes which polymerize polysaccharides or mucins (polysaccharides plus protein). These are enclosed in Golgi vesicles where they polymerize the carbohydrate monomers transported there by membrane activity. The products are secreted as before (fig. 3 at 3). Thus in secretory cells in general we commonly find an abundance of ribosome-covered membranes and an elaborate Golgi apparatus.

Comparative cytological studies have shown that, like the basic biochemistry itself, these intracellular patterns are common to all organisms--with the exception of bacteria which are too small to contain them. In plants protein secretion is uncommon and therefore the ribosome covered ER is scanty. Polysaccharide secretion to form cell wall in contrast is highly developed and is associated with an abundant Golgi apparatus and vesicles containing the polymerized enzymes (pathway 2 and 3 of fig. 3).

An important function in cells is the digestion of food or cellular debris. Food particles enter in vacuoles as at (4) in figure 3 (phagocytosis), and the degradative enzymes, previously bagged up in a vacuole called a lysosome, derived as before from the ER and Golgi, are added to this vacuole to digest its contents. Damaged cellular organelles are bagged up by membranes to form auto-phagocytic vacuoles and are dealt with similarly (fig. 3 at 5). Other enzymes, funneled into tubular branches of the ER, are responsible for lipid and steroid synthesis in cells producing these (fig. 3 at 6). Some enzymes, which serve to transport water or salts across membranes, may find their way to and be adsorbed on extensive meandering, smooth-surfaced tubules, or pleated membranous folds connected with the plasma membrane. Examples are found in kidney cells and in the chloride cells of fish gills.

As mentioned above, differentiated cells elaborate, preferentially, one or another of these functions and the membranous system appropriate to it. The several possibilities, summarized in figure 3, may each individually be developed, with the suppression of other pathways, to become the dominant membranous pattern of a differentiated cell. The characteristic cytological patterns, long familiar at the histological level, thus find their origins and function in the synthesis and transport by their internal membrane system of their most fundamental constituents, enzymes and proteins.

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CHLOROPLAST STRUCTURE AND DEVELOPMENT

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Chloroplasts are particularly good candidates to provide a focal point for molecular biology and agriculture. They are self-replicating subcellular units which can be centrifuged, homogenized, and incubated with all the mixtures beloved by molecular biologists. They appeal to the agriculturalists because increased world food production must ultimately depend upon improving photosynthetic efficiency. This depends upon knowledge of their structure and function.

This paper is concerned with two topics. The first is a discussion of differentiation and senescence in tobacco chloroplasts, and the second revolves around chloroplast specialization in plant groups with unique features of photosynthesis.

The "typical" chloroplast is a disc-shaped organelle about 5-8 μ in diameter. The outer envelope is a double membrane, and a complex series of internal membranes floats in a matrix called

the stroma. The enzymes involved in the dark reaction of photosynthesis are located in this membrane-free fraction. Chlorophyll in chloroplasts is localized within the internal membranes. This system consists of flattened vesicles which are either arranged in stacks called grana, or occur singly and connect the grana. Storage products such as starch, lipid, or protein are found in the stroma.

Chloroplasts are considered to be self-replicating organelles and schemes for their "life cycles" have been proposed (1,2). Developmental sequences can be traced from organelles in the shoot apex to mature chloroplasts in the leaves. The proplastids in the apex have a double membrane, are 1-3 μ in diameter, and possess some stroma lamellae. In addition, they frequently have prolamellar bodies. These are paracrystalline arrays of tubules. In tobacco proplastids, we have also observed single-membrane bound bodies with an electron dense interior. These structures are frequently attached to prolamellar bodies.

The development of the proplastid into a chloroplast parallels the development of the leaf. Both proplastids and partially mature chloroplasts divide. The development is characterized by growth of the plastid and by the differentiation of the internal membrane system. Grana appear both in association with the prolamellar body and with lamellae produced from the inner limiting membrane. In tobacco, grana are also associated with the single-membrane bound body described above. As development proceeds, this body becomes granular and eventually the contents consist of fibrils the size of those shown to be DNA (1). Remnants of the prolamellar body persist until the last stages of tobacco chloroplast development.

Most studies of chloroplast development have utilized dark-grown material which is subjected to light. The organelles in dark-grown leaves which develop into chloroplasts are now frequently called etioplasts. They are usually larger than the proplastids of the stem apex and they usually contain prominent prolamellar bodies. Tobacco etioplasts also contain the single-membrane bodies found in proplastids in light-grown plants. When dark-grown leaves are exposed to the light, the development of chloroplasts is quite rapid. The prolamellar bodies disappear and grana develop. There is a general impression that the prolamellar body is always intimately involved with the formation of grana. This is not always the case, since perfectly good chloroplasts can develop in tobacco tissue cultures even though the etioplasts in dark-grown cultured tissue do not have prolamellar bodies (Laetsch and Stetler, 1965). At present there is a general feeling that the earlier schemes for chloroplast

development are too rigid and do not give recognition to the evidence that there are many ways to make a chloroplast.

Most studies of chloroplast development have stopped with the mature chloroplast in spite of the fact that senescence is a natural part of the developmental sequence. When a leaf senesces on the plant, the most obvious change is bleaching of the green pigment. The initial changes in the chloroplast are the degradation of stroma lamellae. This is followed by increased density and loss of membrane detail in the grana. When tobacco leaves have lost 80 percent of their chlorophyll, the chloroplasts have lost their stroma lamellae and the grana stacks are recognizable as electron-dense clusters. Lipid globules frequently increase during senescence. This natural senescence can be contrasted with a controlled senescence in cultured leaf tissue. Sterile leaf discs of tobacco can be kept alive for many weeks in the dark on a simple mineral-salts-and-sucrose medium. Chlorophyll is lost, but the morphology of the chloroplasts is quite different from those discussed above. The grana disappear, but the stroma lamellae persist. When almost all the chlorophyll is gone, the chloroplasts are without grana. If the leaf discs are transferred at this stage to a medium promoting cell division, the chloroplasts revert to the proplastid condition. Since this tissue will turn green when exposed to light, it is possible to carry a chloroplast through a complete cycle of dedifferentiation and redifferentiation.

The previous description of chloroplast development is more or less typical of a variety of plants like tobacco and bean, but it cannot be said that all chloroplasts are alike. Tropical grasses, such as sugar cane, are a case in point. They have two types of chloroplasts and these dimorphic chloroplasts occur in separate cell layers. The bundle sheath cells surrounding the vascular bundles possess large plastids which do not have grana and which store a lot of starch. The mesophyll cell layer is external to the bundle sheath cell, and it contains "typical" chloroplasts which store very little starch. Some grasses in this general group, such as corn, do have bundle-sheath-cell chloroplasts with rudimentary grana. These dimorphic chloroplasts are of special interest because the grasses possessing them also have a pathway of photosynthetic CO_2 fixation which differs from the Calvin cycle. The primary fixation products in these grasses are C_4-dicarboxylic acids (5). These grasses possess other features in common. They are resistant to photosaturation (2,3); they fail to leak respiratory CO_2 during the light period (4); and they have low CO_2 compensation values (8). In addition, Dr. Bruce Smith (Division of Geology, California Institute of Technology) has shown that they have much higher $^{13}C/^{12}C$ ratios than temperate grasses.

The development of these dimorphic chloroplasts is quite interesting. The proplastids in the meristem at the base of the leaf are very similar even though the two cell layers can be identified. In a portion of the leaf which has 50 percent of its eventual chlorophyll, the plastids in the two cell layers are different, but it is interesting that the bundle sheath cell chloroplasts do have grana at this stage. They are not, however, as well developed as the mesophyll cell chloroplasts. The grana in the bundle sheath cell chloroplasts in the mature cell are a result of reduction and are not plastids with an independent origin. This specialization of chloroplasts has probably followed cell differentiation. What we are really describing is the evolution of an amyloplast or starch-forming plastid.

The etioplasts in dark-grown sugar cane leaves are also different in the two cell layers. When the dark-grown leaves are exposed to light, the prolamellar bodies of the respective etioplasts respond differently, and the bundle sheath cell plastids do not go through an obvious period of grana formation. Short exposures of light induce the etioplasts to form protrusions from their peripheral regions where there is an accumulation of vesicles and tubules. These protrusions have profiles which are very similar to those of mitochondria.

The C_4-dicarboxylic acid pathway of photosynthetic CO_2 fixation found in the tropical grasses has recently been demonstrated in members of the Amaranthaceae and Chenopodiaceae. The leaf anatomies of members of the genera Amaranthus and Atriplex are very similar to each other and to the tropical grasses. Prominent bundle sheaths are surrounded by a layer of palisade cells. The bundle sheath cell chloroplasts are larger than the palisade cell chloroplasts and they store more starch, but they do have grana. The bundle sheath cells of both species contain mitochondria which are much larger than those in the palisade cells. The chloroplasts of these dicots possess a peripheral reticulum similar to that of the tropical grasses and they exhibit protrusions from these areas which are similar to mitochondria. These features have been discussed in detail (6).

The photosynthetic apparatus of these monocots and dicots have much in common and may well represent both morphological and biochemical convergence. There seems to be a trend towards the evolution of plastids in two cell layers which are specialized with respect to the functions of the light and dark reaction of photosynthesis. It might be that there is a greater amount of primary fixation in the mesophyll cell chloroplasts while most of the starch synthesis goes on in the bundle sheath cell chloroplasts. This specialization of labor would result in a more efficient and rapid carbon fixation. There is evidence

that the plants in question evolved in tropical regions where aridity was a problem at some time during the year. An efficient system for carbon fixation would be a definite advantage in regions where water stress would frequently limit photosynthesis. It can be seen how this efficient photosynthetic system has been used by agriculture to great advantage in the humid tropics. More information about the adaptive significance of the behavior of the photosynthetic systems of tropical grasses would provide information which would be useful in future breeding programs.

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SOME ASPECTS OF THE MOLECULAR BIOLOGY OF CHLOROPLASTS

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Studies of a great variety of photosynthetic organisms and higher plants have shown chloroplasts to contain a unique DNA which is different from nuclear and mitochondrial DNA (1). In tobacco, for example, chloroplast DNA constitutes about 10 percent of the total DNA in leaves, amounting to about 5×10^{-15} g. of DNA per chloroplast, has a density of 1.703 vs. 1.698 for nuclear DNA, a minimum mol. wt. of about 4×10^{7} daltons, and in contrast to nuclear DNA, contains no 5-methyl cytosine, and renatures completely after melting (2).

The question of what role chloroplast DNA performs in the development and inheritance of chloroplasts is being investigated by several laboratories throughout the world. In the case of chloroplasts from higher plants, knowledge of the nature of the early stages of chloroplast development is extremely scanty beyond the knowledge that the organelles are not present in seeds and therefore are not transmitted from generation to generation as independent, chlorophyll-containing bodies. However, chloroplast DNA has been identified in seeds (3). Consequently, it is suspected that chloroplast DNA may be the agent which is transmitted and that it contains the molecular information necessary for the synthesis of each new generation of chloroplasts. Support for this view is derived from genetical evidence which has shown that some aspects of chloroplast development are controlled by extranuclear determinants (4).

Chloroplasts contained in the leaves of plants such as tobacco and spinach are complex organelles. When observed in living plant cells, they are seen to be composed of two phases: a stationary component containing a system of lamellae or thylakoids where chlorophyll is located, and a mobile phase (5). The mobile phase is in constant motion and exhibits curious interactions with the mitochondria and cytoplasmic network. Techniques have been developed for isolating chloroplasts with intact mobile phase (6) and for selectively removing the mobile phase from the lamellar system (7). While leaves contain both 80S and 70S ribosomes (8), analysis of the macromolecular composition of the mobile phase of chloroplasts shows it to contain the 70S ribosomes together with 18S Fraction I protein (carboxydismutase) and a mixture of 4-6S proteins. The chloroplast ribosomes have physico-chemical and morphological properties which are closely similar to ribosomes obtained from microorganisms such as E. coli and blue-green algae (8).

Isolated chloroplasts are capable of incorporating amino acids into protein, utilizing polysomes composed of 70S ribosomes (9). About one-half of the total ribosomes in chloroplasts are readily released with the mobile phase, whereas the remainder are tightly bound to the lamellar system so that the lamellae must be broken down by detergents before the ribosomes can be released. Chloroplast DNA is also tightly bound to the lamellar system. In addition to DNA, the lamellar system contains a DNA polymerase which causes incorporation of deoxynucleoside triphosphates into a product identical to chloroplast DNA (10), and an RNA polymerase which utilizes chloroplast DNA as a template for polymerization of nucleoside triphosphates into RNA (11). Histones are not associated with the DNA and thus both the ribosomes and DNA of chloroplasts resemble those in bacteria and serve as an indication that chloroplasts are of ancient origin in the evolutionary scheme of things.

Since chloroplasts appear to have all of the necessary enzymatic machinery which would be required for translating the information in chloroplast DNA into the formation of proteins, the question of how closely these systems are integrated with each other is being intensively investigated. Evidence is being sought as to whether chloroplasts contain specific transfer RNA's different from those in the cytoplasm, whether chloroplast ribosomes can synthesize peptides related to Fraction I protein or other enzymes of the photosynthetic carbon cycle, and whether chloroplast DNA has nucleotide sequences which could code for transfer and ribosomal RNA from chloroplasts. In the case of ribosomal RNA, chloroplast DNA hybridizes with ribosomal RNA from 70S chloroplast ribosomes (0.5 percent) (12,13), but not with RNA from 80S cytoplasmic ribosomes, the latter hybridizing with nuclear DNA (0.3 percent).

Surprisingly, chloroplast ribosomal RNA also hybridizes with nuclear DNA (0.1 percent). In fact, the informational content of nuclear DNA related to chloroplast ribosomal RNA is about three times greater than that contained in chloroplast DNA since the latter constitutes only 10 percent of the total cellular DNA. On a per nucleus per chloroplast basis, the information in the nucleus capable of coding for chloroplast RNA is about 1000 times greater than that contained in the DNA of a chloroplast. There appear to be about 800 cistrons in nuclear DNA complementary to chloroplast ribosomal RNA, compared to only about 8 cistrons in chloroplast DNA. Apparently, only about 10 percent of the chloroplast DNA contains nucleotide sequences complementary to chloroplast ribosomal RNA suggesting that much of the potential information in chloroplast DNA could code for other purposes. It has been estimated (1) that the DNA in a single chloroplast would be sufficient to code for about 1600 different proteins, each containing 200 amino acids in the primary structure of their subunits.

Is the information in nuclei for chloroplast ribosomal RNA utilized during the development of chloroplasts or is it a nonfunctional relic of past evolution? There is evidence that nuclear and chloroplast DNA's share common nucleotide sequences as detected by hybridization (10,14). Perhaps the information required for a photosynthetic apparatus was contained in a primeval nuclear DNA which became separated as a distinct chloroplast DNA at the time in evolution when the photosynthetic apparatus became enclosed within an organelle distinct from the nucleus. On this basis, it would be no surprise to still find redundancy in the form of nuclear DNA sharing some base sequences in common with chloroplast DNA. However, it seems certain that nuclear DNA does have a role in the development of chloroplasts as attested by the many nuclear mutants which affect chlorophyll biosynthesis and other aspects of chloroplasts (4).

Some insight into the problem of the role of nuclear DNA in chloroplast development may come from investigation of a mutant which results in a random variegation of tobacco leaves. Cells in the variegated areas contain mixed populations of normal and defective chloroplasts, the latter being devoid of chlorophyll. The defective chloroplasts appear as if they were composed only of mobile phase with little or no lamellar systems. The defective chloroplasts contain a normal amount of chloroplast DNA as well as an active RNA polymerase, together with a normal amount of 70S chloroplast ribosomes. As a working hypothesis, it might be surmised that the information necessary for the macromolecular constituents of the mobile phase is provided by nuclear DNA. In this view, chloroplast DNA may contain the information necessary for the formation of the lamellar system, and a defect in the

chloroplast DNA may prevent synthesis and programming of a special class of chloroplast ribosomes required for synthesis of the lipoproteins of the lamellar system.

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BACTERIAL SPORULATION: A MODEL SYSTEM FOR THE MOLECULAR APPROACH TO MORPHOGENESIS

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Bacterial sporulation is an example of unicellular morphogenesis in which a metabolically active, rod-shaped vegetative cell is converted to a dormant, ellipsoidal spore. The two forms differ not only in morphology and metabolic activity but in several other properties, including water and metal content, cell-wall structure and proteins, heat and chemical resistance, presence of dipicolinic acid, and protein and nucleic acid content. In fact, bacterial spores represent an interesting case of morphogenesis and an extreme case of biological dormancy, since they may remain dormant for at least 250 years.

Sporulation is a favorable system for analyzing morphogenesis, because it can be approached by biochemical, cytological, and genetic techniques. Furthermore the different morphological stages are very distinct. Studies by many investigators have shown that a sequence of biochemical and physiological events occurs during spore formation (1-3). Mutants have been isolated which develop to particular points in this sequence (4). Since RNA fractions play a key part in translating the genetic message during protein synthesis and may be involved in regulating the expression of specific genes, our approach has been to characterize the RNA fractions from vegetative cells, sporulating cells, and dormant spores of Bacillus subtilis. The goal has been to identify changes in the RNA patterns and to correlate them with specific functions during morphogenesis.

Studies on bulk RNA. Sporulation occurs after active growth has terminated. During sporulation the net synthesis of nucleic acid ceases; however, an active turnover of all RNA species occurs (5). There is a preferential breakdown of ribosomes (6), resulting in an increasing ratio of transfer RNA (tRNA) to ribosomal RNA (rRNA) during spore formation (7). The tRNA-to-rRNA ratio reaches its maximum in the spore. This relationship between the tRNA and rRNA is related to the metabolic activity of the cell, since there is an inverse relationship between the tRNA/rRNA ratio and growth rate of B. subtilis. The RNA content of spores is usually one-half that of vegetative cells.

Studies on messenger RNA (mRNA). If the occurrence of new proteins during sporulation is dependent on transcriptional control, mRNA synthesis should be affected. Sporulating cells contain a unique mRNA fraction in addition to the mRNA found in actively

growing cells. This has been shown by DNA-RNA hybrid competition studies (8,9). These results suggest that a general repression of genes active in vegetative cells does not occur; however specific genes are derepressed for sporulation functions. The fact that mRNA of germinating spores is similar to that of active vegetative cells indicates that genes active during sporulation are repressed once again during germination. No evidence has been found for the presence of mRNA in dormant spores of B. subtilis.

Studies on ribosomes and ribosomal RNA. Dormancy could be a function of some defective component in the protein-synthesizing machinery. A comparison of the ribosome fractions of spores and vegetative cells has revealed no significant differences in their physicochemical properties (10). The RNA and protein contents and the sedimentation properties of the ribosomes are identical. Spore ribosomes are able to bind synthetic polynucleotides and aminoacyl-tRNA to almost the same extent as vegetative cell ribosomes. The base composition and sedimentation coefficients of rRNA are very similar. DNA-rRNA hybrid competition studies have shown that rRNAs from spores and vegetative cells have identical or nearly identical base sequences. These results suggest that the ribosomal components of the sporulating cell are enclosed intact into the developing spore.

Studies on transfer RNA. Recent investigations have suggested that tRNAs may play a role in regulating the translation process. Therefore the elution profiles of tRNA from vegetative cells and sporulating cells were compared by co-chromatography through a methylated albumin kieselguhr column to determine whether significant changes occurred in their patterns. Most of the aminoacyl-tRNA patterns remained constant in these analyses. However, a significant change in the valine tRNA pattern was observed in early sporulation cells (11). The alteration in the pattern consisted of an increase of one of the two valine tRNA species. A more extensive study has revealed that a similar alteration occurs during step-down and step-up growth transitions and during the stationary phase of an asporogenous mutant. We are investigating this change in the ratio of two valine tRNA species to determine whether it is due to differential synthesis or to some type of activation. Also it is of interest to determine whether a direct relationship exists between tRNA pattern changes and sporulation or particular growth conditions.

The tRNAs of spores are capable of accepting amino acids (12,13). Aninoacyl-tRNA synthetase activity has also been demonstrated in spore extracts. This part of the translation mechanism appears to be intact in dormant spores.

Summary. The sporulating bacterium Bacillus subtilis is a highly suitable system for analyzing RNA changes and functions during morphogenesis. An active turnover of RNA is noted during spore formation. Qualitative differences in the messenger RNA population are observed, indicating the expression of genes specific for sporulation. Changes in the pattern of valine transfer RNA during sporulation are being analyzed to determine whether they are due to differential synthesis or modification and whether they have a direct relationship to regulation of gene expression. Although degradation of ribosomes occurs during spore formation, the spore ribosomes appear to be normal. In fact all components of the protein synthesizing machinery of spores analyzed to date appear to be functional, suggesting that dormancy is not caused by a defect in the translation mechanism.

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NUCLEIC ACIDS IN EVOLUTION, TAXONOMY AND DEVELOPMENT

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Reactions involving the formation of duplexes, either DNA/DNA of DNA/RNA, are in current use in many areas of biological investigation. By virtue of their great specificity these reactions provide a means for comparisons of nucleotide sequences in nucleic acids of related organisms (1). They provide a rapid and convenient means for making such comparisons without the actual determination of base sequence. The cross-reaction of two DNA single strands from different organisms is a measure of the amount of divergence in base sequence which has taken place in the evolution of these organisms (2). This has proved useful for tracing the evolution of various groups of organisms from viruses to primates at the molecular level as well as a quantitative taxonomic method.

Similar methods are useful for the analysis of gene transcription through the formation of DNA/RNA hybrids (3). This methodology offers an approach to the analysis of differential patterns of gene expression in development (4).

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LEAF ABSCISSION: THE CHRONOLOGY AND CONTROL OF A TERMINAL DEVELOPMENTAL SEQUENCE

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Leaf abscission can be initiated by senescence, injury, disease, environmental changes or chemical defoliants. The developmental sequence for bean (Phaseolus vulgaris L.) leaf abscission, a widely used experimental system, is visualized as including: (a) Appearance of chemical messengers that result in induction of synthesis of specific informational RNA through regulation of the template activity in specific regions of the DNA; (b) Synthesis of abscission-specific enzymes, including cell-wall-dissolving enzymes, under direction of the newly formed informational RNA; (c) Secretion of wall-dissolving enzymes into cell walls adjacent to the separation layer; (d) Dissolution of cell walls within the separation layer, which continues until some minimum-break strength is achieved; and (e) Separation aided by internal shear forces generated by differential growth and/or hydrostatic pressure.

For in vitro studies, explants (12), 1.0 to 1.5 cm. long, are cut from unifoliate (primary) leaves of ca. 18-day-old bean plants and placed upright (petiole or pulvinar end down) in 1 percent agar in closed petri dishes. These explants contain the abscission zone at the leaf blade (pulvinus)-petiole juncture. Separation (abscission) of 50 percent of the explants occurs in about 100 hours. In comparison, natural abscission of unifoliate leaves occurs when plants are 30 to 40 days old. In vitro abscission experiments with plants other than bean are conducted in a similar manner.

Morphological studies emphasize that cell-wall breakdown is an important aspect of cell separation (4,12). Bean leaf abscission occurs primarily by dissolution of cell contacts between adjacent tissue regions.

Prior to separation layer formation, the abscission zone is not a point of structural weakness (9). As determined with an Instron linear stress-strain analyzer, formation of the separation layer is indicated by a marked decline in break strength across the abscission zone. With explants, the decline is linear for the first 48 to 72 hours, from a maximum of ca. 500 g at t=0 to a minimum of 50 to 100 g when the separation layer is complete.

Formation of this separation layer is retarded by inhibitors of RNA (actinomycin D, 5-fluoroduracil) and protein (cycloheximide, puromycin) synthesis (2,3,6) and accelerated by ethylene and compounds that promote ethylene production (1). As development proceeds, the abscission processes become less sensitive to inhibitors and more responsive to ethylene.

When abscission is accelerated by ethylene, there is an accompanying increased rate of RNA synthesis (P^{32} incorporation) followed by an increased rate of protein synthesis (leucine incorporation) (2,3,6). Incorporation of P^{32} into all classes of RNA is accelerated, but the greatest increases in specific activity are associated with the messenger fractions (6). These changes in RNA and protein synthesis are restricted to the abscission zone and adjacent petiole-derived cell layers as observed through use of histochemistry and autoradiography (Barbara Webster and A. C. Leopold, private communication). During the 24-hour period following deblading and excission, patterns of H^3-leucine and -uridine incorporation as well as size and staining of nucleoli are correlated with separation layer formation and accelerated by application of ethylene. Affinity of nuclei for fast green (5), 1 percent pH 8.0 to 8.1 (for basic proteins such as histones) increases during the first 24 hours in the pulvinus cells of bean explants and then remains constant (Wederitsch and Morré, unpublished). These observations are consistent with a "step up"

transition in the abscission zone and adjacent petiolar cells prior to separation layer formation and a "step down" transition in pulvinar cells preceding their senescence and death.

Except for rupture of the vascular strands, wall changes leading to separation layer formation are complete in 72 hours. Chemical analyses of abscission zones reveal small quantitative changes (5 to 20 percent) in arabinose, galactose, and galacturonic acid of the classical pectin fraction and an increase in the hot- and cold-water-extractable pectin fractions at the expense of the pectin fraction extractable with dilute mineral acid (9). These results are consistent with wall dissolution through hydrolysis of glycosidic linkages catalyzed by the action of endopolygalacturonases.

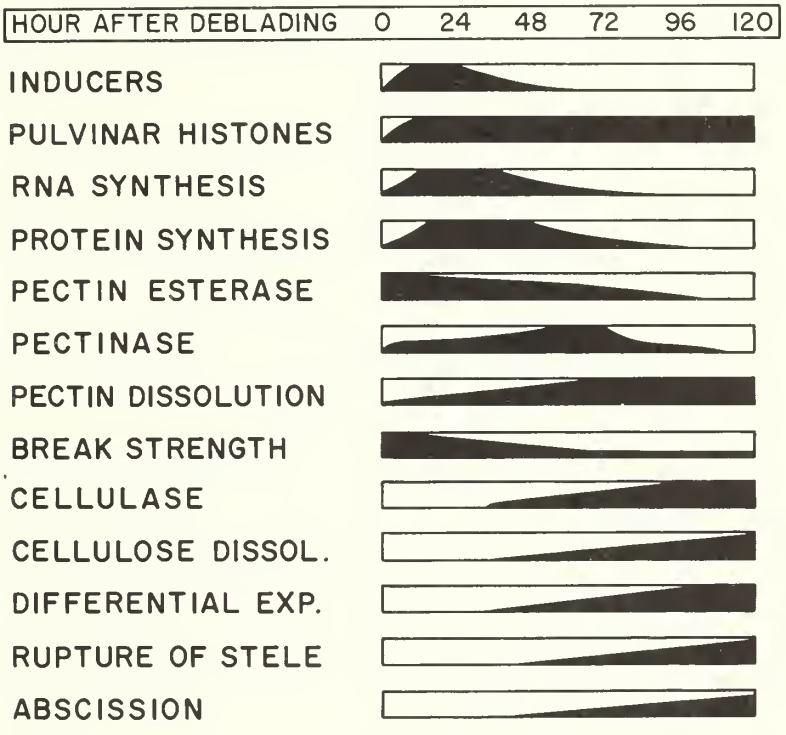

Figure 1. Probable chronology of events during abscission of explants prepared from the distal abscission zone of unifoliate bean (Phaseolus vulgaris) leaves.

The appearances of endopolygalacturonases coincides with wall dissolution and precedes cell separation. As determined by a sensitive and specific bioassay (H. W. Mussell and D. J. Morré, in preparation), polygalacturonase activity increases from no detectable enzyme at t=0 to a maximum of ca. 0.01 µg. per abscission zone at 72 hours (compared with ca. 10 µg. polyanhydrogalacturonic acid per abscission zone). Results with neutral red staining and concomitant plasmolytic studies

suggest that the protoplasts remain intact during cell separation and that the enzymes responsible for wall dissolution are secreted. Changes in pectinesterase (10,11), cellulases (7), and divalent ions (11) are associated with abscission in beans, all of which might contribute to cell-wall softening. However, an area of structural weakness at the abscission zone is obtained in vitro by treatment of half-explants with purified endopolygalacturonases, and secretion of these enzymes appears both necessary and sufficient for the separation layer to form in beans. A projected chronology of abscission events in bean explants is summarized in figure 1.

What molecules initiate the rise in pectinase and other wall dissolving enzymes? In fungi (8), pectin, galacturonic acid and galactose act as endopolygalacturonase inducers. When applied to bean explants, galactose and galacturonic acid markedly accelerate abscission. The next step is to relate these effects to levels of enzymes in bean cell walls.

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METABOLIC AND PHYSIOLOGICAL DEVELOPMENT IN PLANT TISSUES

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Thin slices of fleshy storage organs serve as an effective prototype system for biochemical ontogeny and differentiation in plant tissue. Immediately upon cutting, a train of biochemical events is evoked which results in the development of a respiratory increment which is qualitatively distinct from the initial, or basal, respiration, and which is associated with a sharp increase in phosphorylative activity, and a spate of physiological activity which depends on oxidative phosphorylation. Insofar as the transformation at issue is confined to a few layers of surface cells, it has been proposed that one parameter which controls the metabolic condition of a cell is its spatial relationship to its neighbors and to the environment. Thus, the metabolism of cells or tissues behind the growing point of root or shoot may change with the distance of the cell or tissue from the surface--and such appears to be the case.

The developed respiratory rise in slices, as well as the associated physiological changes, are prevented by actinomycin and by puromycin. Whereas the respiration rise develops through 24 hours or longer, actinomycin and puromycin inhibit the rise

only if presented during the first 10 hours. While actinomycin interferes with uracil incorporation into RNA, and puromycin inhibits leucine incorporation into protein, throughout the full 24-hour period, what has been termed reciprocal inhibition--i.e., the inhibition or uracil incorporation by puromycin and leucine incorporation by actinomycin--is evidenced only in the first 10 hours. The coincident time-course and extent of reciprocal inhibition evoked by puromycin and actinomycin respectively have suggested ribosome synthesis as the primary event leading to the multitude of the observable changes. Experiments wherein uracil-2-C^{14} was presented in successive 4-hour intervals, and its incorporation into ribosomal RNA determined, have indicated that ribosome synthesis is marked immediately after slicing, and drops to a low value in 12 hours. Puromycin-sensitive RNA synthesis is entirely accounted for by ribosomal RNA synthesis. Thus newly synthesized ribosomes, and not new messenger RNA alone, underlie the metabolic changes which follow slicing.

The metabolic block in fresh slices appears to center on isocitric dehydrogenase. However, while fresh slices show no overall tricarboxylic acid cycle activity, mitochondria from fresh tissue as normally isolated implement each step of the cycle. Methods have been developed to prepare mitochondria morphologically akin to mitochondria in vivo, with the end in view of ascertaining whether mitochondria from fresh tissue, which have not been altered morphologically during isolation, display a relatively inactive isocitric dehydrogenase. Filiform mitochondria have been prepared by the addition of any of a number of large-molecular-weight substances to the preparative medium, and such filiform mitochondria are reversibly transformable to spherical forms, without swelling. An examination of the isotherm for isocitrate oxidation by both filiform and spherical mitochondria, from fresh and aged tissue, points to the likelihood that mitochondrial isocitric dehydrogenase is under allosteric control in potato. It remains to establish with certainty whether the ultimate consequence of the complex and extensive biochemical events which are initiated by slicing is the unfettering of isocitric dehydrogenase.

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